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PLENA and *FARINELLI*: redundancy and regulatory interactions between two *Antirrhinum* MADS-box factors controlling flower development

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We report the discovery of an *Antirrhinum* MADS-box gene, *FARINELLI* (*FAR*), and the isolation of *far* mutants by a reverse genetic screen. Despite striking similarities between *FAR* and the class C MADS-box gene *PLENA* (*PLE*), the phenotypes of their respective mutants are dramatically different. Unlike *ple* mutants, which show homeotic conversion of reproductive organs to perianth organs and a loss of floral determinacy, *far* mutants have normal flowers which are partially male-sterile. Expression studies of *PLE* and *FAR*, in wild-type and mutant backgrounds, show complex interactions between the two genes. Double mutant analysis reveals an unexpected, redundant negative control over the B-function MADS-box genes. This feature of the two *Antirrhinum* C-function-like genes is markedly different from the control of the inner boundary of the B-function expression domain in *Arabidopsis*, and we propose and discuss a model to account for these differences. The difference in phenotypes of mutants in two highly related genes illustrates the importance of the position within the regulatory network in determining gene function.

Keywords: floral determinacy/homeotic mutants/MADS-box/male sterility/regulatory network

Introduction

The study of *Antirrhinum* and *Arabidopsis* floral homeotic mutants produced a simple combinatorial model to explain how the regulated expression of a few genes could determine the organ composition of the flower (Coen and Meyerowitz, 1991). The model proposes that three functions are expressed in adjacent, overlapping whorls such that the A-function is expressed in whorls 1 and 2, the B-function in whorls 2 and 3, and the C-function in whorls 3 and 4. Expression of the A-function alone determines sepal development, co-expression of the A- and B-functions or the B- and C-functions specifies petals or stamens, respectively, and expression of the C-function alone results in carpel development. In B-function mutants, petals and stamens are replaced by sepals and carpels, respectively. In C-function mutants, perianth organs develop in place of reproductive organs. In both *Antirrhinum* and *Arabidopsis*, a single C-function and two B-function genes have been identified and shown to encode MADS-box transcription factors (Sommer *et al*., 1990; Yanofsky *et al*., 1990; Jack *et al*., 1992; Tröbner *et al*., 1992; Bradley *et al*., 1993; Goto and Meyerowitz, 1994).

The MADS-box family is defined by the presence of a 58 amino acid domain which is conserved between the founding members; MCM1 (yeast), AGAMOUS and DEFICIENS (plants) and SRF (animals) (Schwarz-Sommer *et al*., 1990). Although MADS-box genes have been identified and characterized in animals and yeasts (Shore and Sharrocks, 1995), they are found most extensively in plants (Theissen *et al*., 1996, Liljegren *et al*., 1998). The >200 known plant MADS-factors, from many different species, have been divided into sub-families based mainly on their predicted amino acid sequences (Doyle, 1994; Purugganan *et al*., 1995; Theissen *et al*., 1996). Often, genes from different species which fall into one of these sub-families have similar expression patterns and similar roles in development. For example, the B-function genes of *Antirrhinum* [*DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*)] and *Arabidopsis* [*APETALA3* (*AP3*) and *PISTILLATA* (*PI*)] are expressed mainly in the second and third whorls and show similar mutant phenotypes. Similarly, the C-function genes of *Antirrhinum* [*PLENA* (*PLE*)] and *Arabidopsis* [*AGAMOUS* (*AG*)] are expressed in the third and fourth whorls and show similar mutant phenotypes. Currently identified functions of the MADS-box gene family include the control of organ identity (see above), flowering time (Sheldon *et al*., 1999), meristem identity (Huijser *et al*., 1992; Mandel *et al*., 1992; Kempin *et al*., 1995), regulation of ovule development (Angenent *et al*., 1995), regulation of fruit development (Gu *et al*., 1998) and control of root architecture (Zhang and Forde, 1998).

Here we report the isolation of a novel *Antirrhinum* MADS-box gene, *FARINELLI* (*FAR*), and a recessive *far* mutant. We show that all our current criteria identify *FAR* as a second C-function gene. However, *far* mutants display none of the typical features of C-function mutants and instead show reduced male fertility. Furthermore, the *ple*/*far* double mutant reveals that *PLE* and *FAR* act redundantly to prevent expression of the B-function genes, *DEF* and *GLO*, in the fourth whorl. We propose a model for the interactions of *PLE* and *FAR* and show that two crucial differences could account for the variation in single and double homeotic mutant phenotypes observed in *Antirrhinum* and *Arabidopsis*.

Results

Isolation of a second Antirrhinum C-function gene

To identify further members of the MADS-box gene family, an *Antirrhinum* floral cDNA library was screened

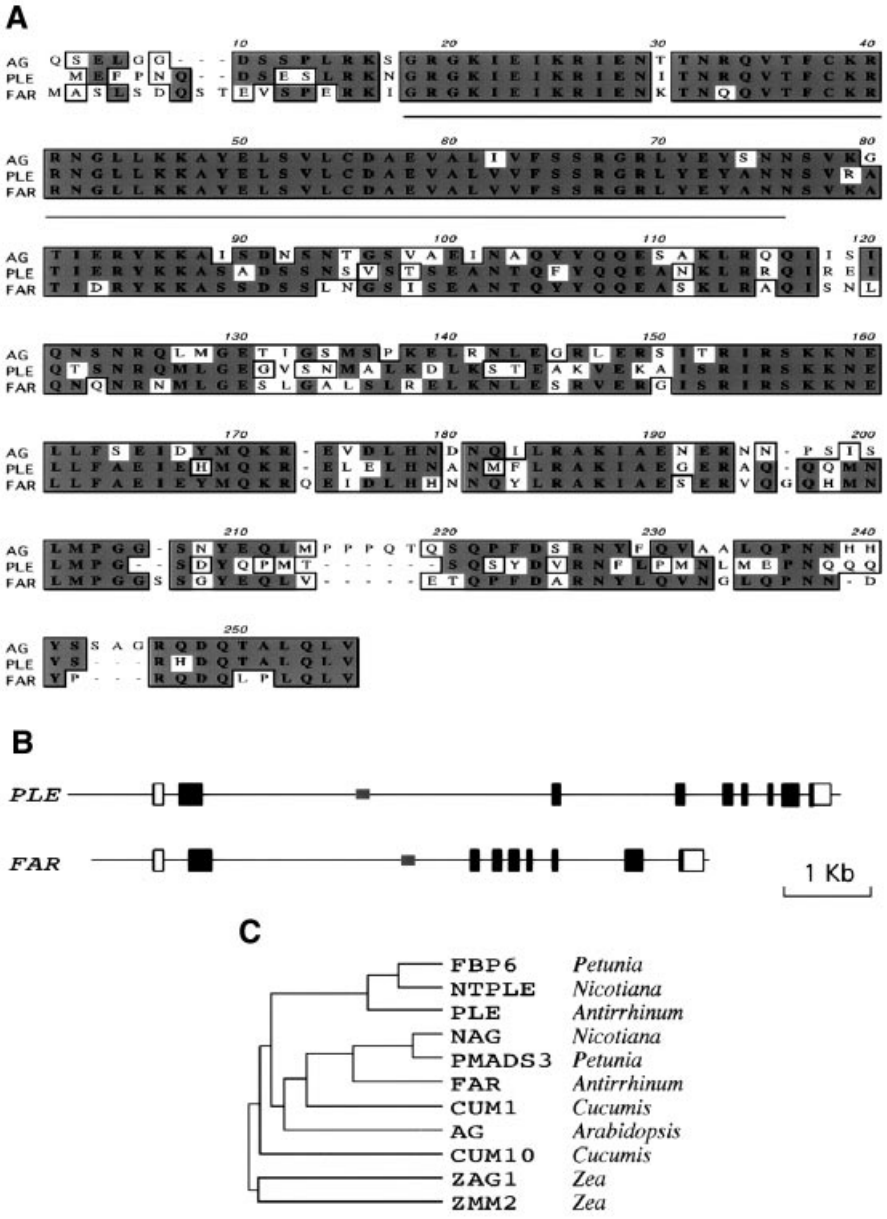

Fig. 1. Sequence analysis of FAR. (**A**) The predicted amino acid sequences of AG, PLE and FAR were aligned using MacVector. For the purposes of this alignment, AG was truncated at its N-terminal end. Identical residues are shown boxed and shaded. Gaps have been introduced to maximize alignment. The MADS-box is underlined. MADS-box factors which are known to control similar processes in different species have been noted to share a short stretch of amino acid identity at their C-terminal ends (Hansen *et al*., 1993). AG, PLE and FAR share the sequence LQLV in this position. (**B**) Schematic diagram of the exons (boxes) and introns (lines) of the *PLE* and *FAR* genes. Shading within the boxes signifies coding regions. The information concerning *PLE* was compiled from Bradley *et al.* (1993) and from our sequence data (B.Davies and H.Sommer, unpublished). The small shaded boxes in the second introns signify the position of the conserved sequence referred to in the text. (**C**) C-function factor sequences from *Antirrhinum* (Bradley *et al*., 1993), *Arabidopsis* (Yanofsky *et al*., 1990), petunia (Tsuchimoto *et al*., 1993; Angenent *et al*., 1994), tobacco (Kempin *et al*., 1993), cucumber (Kater *et al*., 1998) and maize (Mena *et al*., 1996) were collected and analysed by the GCG pileup program (Devereux *et al*., 1984).

using the *Arabidopsis* C-function gene *AG* (Yanofsky *et al*., 1990) as a probe. Sixteen strongly hybridizing clones which did not correspond to *PLE* were identified, and DNA sequence analysis showed that they were derived from the MADS-box gene, *FAR*, which is closely related to, but distinct from, *PLE* (Bradley *et al*., 1993) (Figure 1A). A *FAR* probe was used to screen an *Antirrhinum* genomic library and the DNA sequence of 7 kb spanning the *FAR* coding region was determined. The *FAR* gene, like *PLE*, comprises nine exons, the first of which is non-coding (Figure 1B). In both cases, the MADS-box is encoded by the second exon and is preceded by 16 or 17 amino acids (Figure 1A). This N-terminal extension is a characteristic of C-function homeotic MADS-box factors, such as PLE and AG, and related proteins such as AGL1 and AGL5 (Ma *et al.*, 1991). Within the MADS-box, each pair of factors is 95% identical and, over the entire protein sequence there is 64–67% identity (Figure 1A). Thus, based on the functions of the most closely related genes, it appeared likely that FAR is a C-function factor involved in determining the identity of the floral reproductive organs. Another feature of the C-function genes, which is

also shared by *FAR*, is the presence of a large intron after the MADS-box-containing second exon (Figure 1B). There is evidence to suggest that this intron contains regulatory elements which contribute to the control of spatial and temporal expression of *PLE* and *AG* (Bradley *et al*., 1993; Sieburth and Meyerowitz, 1997). We note the presence of a 70 bp sequence (3581–3650 in the *FAR* genomic sequence), including a direct repeat of the sequence CCAATCA, which is 60% identical between sequences in this intron of *FAR* (AJ239057), *PLE* (D.Bradley, R.Carpenter and E.Coen, unpublished), *AG* (ATF13C5) and *PTAG1* (AF052570), suggesting a possible role for this sequence in the negative regulation of C-function gene expression (Figure 1B).

Two types of *FAR* cDNA were identified in the cDNA library at different frequencies. The DNA sequence and predicted amino acid-coding potential of the rarer of the two cDNAs was identical to the more common cDNA except for an insertion of four codons (QQLF inserted between amino acids 103 and 104). Comparison of the cDNA and genomic sequences revealed that the rarer variant is derived from differential splicing which makes use of an alternative splice acceptor site within the third intron. PCR analysis of this region from the 16 independent *FAR* cDNA clones showed that four utilized the alternative splice site. The variation occurs within a region of the MADS-box protein known as the I-domain (Ma *et al*., 1991), the length and sequence composition of which have been postulated to affect the heterodimerization specificity amongst MADS-box transcription factors (Huang *et al*., 1996). It is not known what, if any, function is served by the inclusion of an additional four amino acids in a minor variant of FAR, but it remains possible that the presence of two forms of FAR alters its heterodimerization potential.

Two C-function-like genes have been isolated from several species including *Antirrhinum* (Bradley *et al*., 1993; this report), petunia (Angenent *et al*., 1993; Tsuchimoto *et al*., 1993), tobacco (Kempin *et al*., 1993; H.Sommer, unpublished), cucumber (Kater *et al*., 1998) and maize (Schmidt *et al*., 1993; Theissen *et al*., 1995; Mena *et al*., 1996). In petunia, tobacco and *Antirrhinum*, the two C-function-like factors form sub-families; FBP6, NTPLE and PLE form one sub-family and NAG, PMADS3 and FAR form the other (Figure 1C). AG is most closely related to the FAR family. One of the two cucumber factors, CUM1, falls into the same sub-family as AG, whereas the other, CUM10, does not fit into either sub-family. It has been suggested that the two maize genes *ZAG1* and *ZMM2* are members of a split C-function, each performing a separate role in floral organ development (Mena *et al*., 1996). However, since they are more related to each other than to members of the two dicot sub-families, it is likely that a split C-function evolved independently in maize. Discussion of the apparent implications of these observations for the evolution of class C genes and the splitting and/or switching of their role in the control of different aspects of the C-function is beyond the scope of this report. We shall focus rather on developmental implications, since *Antirrhinum* is the first species in which mutants for both C-function genes have been identified and where the contribution of each gene to the C-function can be studied independently and in combination.

Comparison of PLE and FAR expression patterns

Initial Northern blot and *in situ* hybridization experiments suggested that the expression patterns of *PLE* and *FAR* were identical, in that both are expressed in the third and fourth whorls (not shown). However, more detailed analysis, using sequential sections reveals slight differences in their expression patterns. At stage 4, when expression of *PLE* first becomes apparent (Bradley *et al*., 1993), the expression patterns of *PLE* and *FAR* are very similar (Figure 2A and C). Differences become apparent by stage 6 when *PLE* expression is detected in the developing stamen and the carpel primordia (Figure 2B). *FAR* expression is also observed in these tissues and at the lower border of *PLE* expression in a bowl-shaped region linking the stamen primordia (Figure 2D). At late stages of development, *PLE* and *FAR* show distinct expression patterns (Figure 2E and F). In the anther, *PLE* transcripts become localized to the region of the stomium (arrowed in Figure 2E) whereas *FAR* is expressed predominantly in the connective. In the gynoecium, *PLE* is expressed mainly in the developing ovules and to a lesser extent in the placenta and carpel wall (Figure 2E). In contrast, *FAR* expression is weaker in the developing ovules and stronger in the placenta (Figure 2F).

To investigate the possibility that *PLE* controls the expression of *FAR*, *in situ* hybridization experiments were carried out using two *PLE* mutant alleles; *ple*-1, a loss-of-function *ple* mutant, and *Ple*-888, a gain-of-function *ple* mutant in which *PLE* is ectopically expressed. At stage 6, *PLE* expression, which is confined to whorls 3 and 4 in wild-type flowers (Figure 2B), is detected strongly in all whorls of the *Ple*-888 mutant (Figure 2G). *PLE* expression can be seen on the inner surface of the organs of the first whorl (which will become carpeloid) and in the second whorl organs (which will become staminoid). In contrast, at this stage, little *FAR* transcription can be detected in the outer whorls of *Ple*-888 mutant flowers (Figure 2H). In mature *Ple*-888 flower buds, *PLE* transcripts are still detected in all floral organs (Figure 2I), and ectopic expression of *FAR* mimics that of *PLE* (Figure 2J). Thus, ectopic *FAR* expression is induced by ectopic expression of *PLE*, but there is a delay in the induction of *FAR*. This delay could indicate that the induction of *FAR* by *PLE* is indirect and relies on a *PLE*-dependent alteration of organ identity, or that *PLE* can only induce *FAR* expression in combination with other, later expressed factors. Alternatively, induction of *FAR* expression could require higher levels of PLE than are present at early stages of development of the outer organs in *Ple*-888 mutants.

That *PLE* expression is not absolutely required for the induction of *FAR* can be seen by studying the transcription of *FAR* in *ple* mutants. *ple*-1 flowers show all the characteristics of a loss of C-function: reproductive organs are replaced by perianth organs and the flower becomes indeterminate and produces many internal whorls of organs (Figure 3F and G). Although these flowers have no stamens or carpels, reduced *FAR* expression can be detected in all whorls internal to the second (Figure 2K and L). *ple*-1 is not a null allele and occasionally produces a small

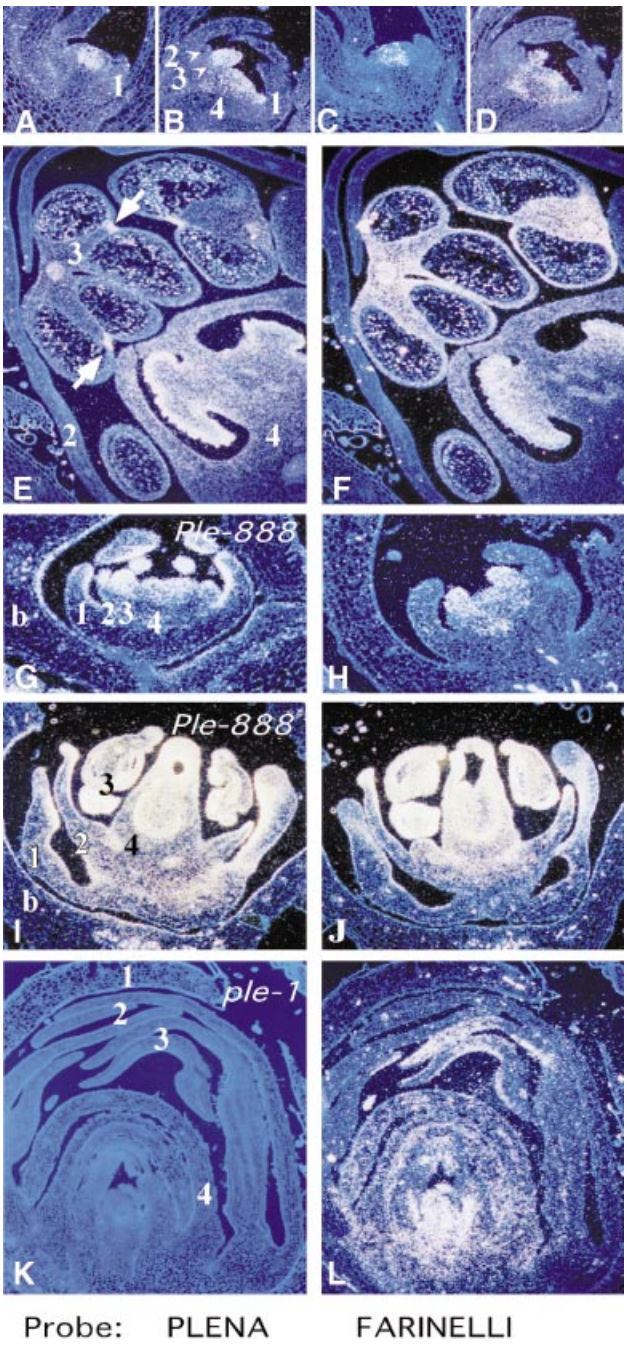

Fig. 2. *In situ* analysis of *PLE* and *FAR* expression in wild-type and mutant flowers. Sections in the left hand column, with the exception of (K), were probed with *PLE* (A, B, E, G and I). All sections in the right hand column were probed with *FAR* (C, D, F, H, J and L). Serial sections of stage 4 (**A** and **C**), stage 6 (**B** and **D**) and mature (**E** and **F**) wild-type floral buds are shown. Sections of *Ple*-888, a semi-dominant mutant in which *PLE* is ectopically expressed, are shown at stage 6 (**G** and **H**) and mature stages (**I** and **J**). Sections through a mature flower bud of the recessive *ple*-1 mutant are shown unhybridized (**K**) and hybridized with *FAR* (**L**). Developmental stages were defined according to Carpenter *et al*. (1995) and Zachgo *et al*. (1995). Whorls are numbered and bracts (b) are indicated where appropriate.

amount of pollen on the third whorl petals. In a *ple* null mutant background, *FAR* expression is reduced further, but not abolished (not shown). Thus expression of *FAR* is not dependent on *PLE* although, directly or indirectly, *PLE* is required for full expression of *FAR* and can induce ectopic *FAR* expression at later stages.

Protein–protein interactions

The similarities between *FAR* and other C-function genes suggested a role in the determination of reproductive organ identity. However, the fact that *FAR* expression is observed in *ple* mutants lacking reproductive organs indicated that *FAR* cannot substitute for *PLE*. To discover a molecular basis for the failure of two such similar proteins to provoke the same developmental response, we analysed their protein–protein interactions as detected in a yeast two-hybrid assay. We previously had identified four MADS-box factors (SQUA, DEFH49, DEFH200 and DEFH72) that interact with PLE (Davies *et al*., 1996b). A similar screen was carried out with full-length FAR as bait. Ten colonies which passed all the controls were identified from 3.5×10^6 primary transformants (Table I). Eight of the positives corresponded to *DEFH200*, one to *SQUA* and one to *DEFH49*. As was the case with the screens involving PLE, we did not identify any other interacting MADS-box factors, such as those of the B-function, and neither did we identify PLE or FAR as FAR-interacting proteins. Subsequent cross-testing of interactions by introducing pairs of cloned MADS-box genes in the appropriate vectors into yeast also failed to find any evidence for interaction between FAR and FAR, PLE, DEF, GLO or DEFH72, although they confirmed the interactions with DEFH200, SQUA and DEFH49 (Table II).

These experiments suggest that differences in the activity of PLE and FAR are unlikely to result from absolute differences in their potential to interact with other MADS-box factors, unless the apparent inability of FAR to interact with DEFH72 makes a significant difference to its ability to induce developmental changes. Further experiments will be required to determine if this differential interaction is responsible for the difference in activity. Although only a single potential difference in protein–protein interactions was detected, subtle alterations in the stability of the heterodimers formed between the common partners and FAR or PLE and/or in their DNA-binding affinity or specificity cannot be excluded.

Ectopic expression analysis in transgenic plants

As a first step to identify a function for the *FAR* gene in wild-type flower development, it was expressed under the control of a constitutive promoter, in tobacco plants. It has been shown previously that 35S::*PLE* tobacco plants show the typical features of ectopic C-function expression (Davies *et al*., 1996a). The plants are normal until flowering, when they produce abnormal flowers with carpeloid organs in the first whorl and stamenoid organs in the second whorl, although the homeotic conversion of the second whorl organs is less complete than that of the first whorl organs. Figure 3B shows a typical flower of a 35S::*PLE* tobacco plant with a dramatic conversion of the first whorl sepals towards carpeloidy (compare with wild-type flower in Figure 3A). In contrast, the first whorl sepals of 35S::*FAR* flowers are often completely normal, but sometimes grow to be slightly larger than is usual in the wild-type and show slight carpeloidy. The organs of the second whorl show a much more complete transition

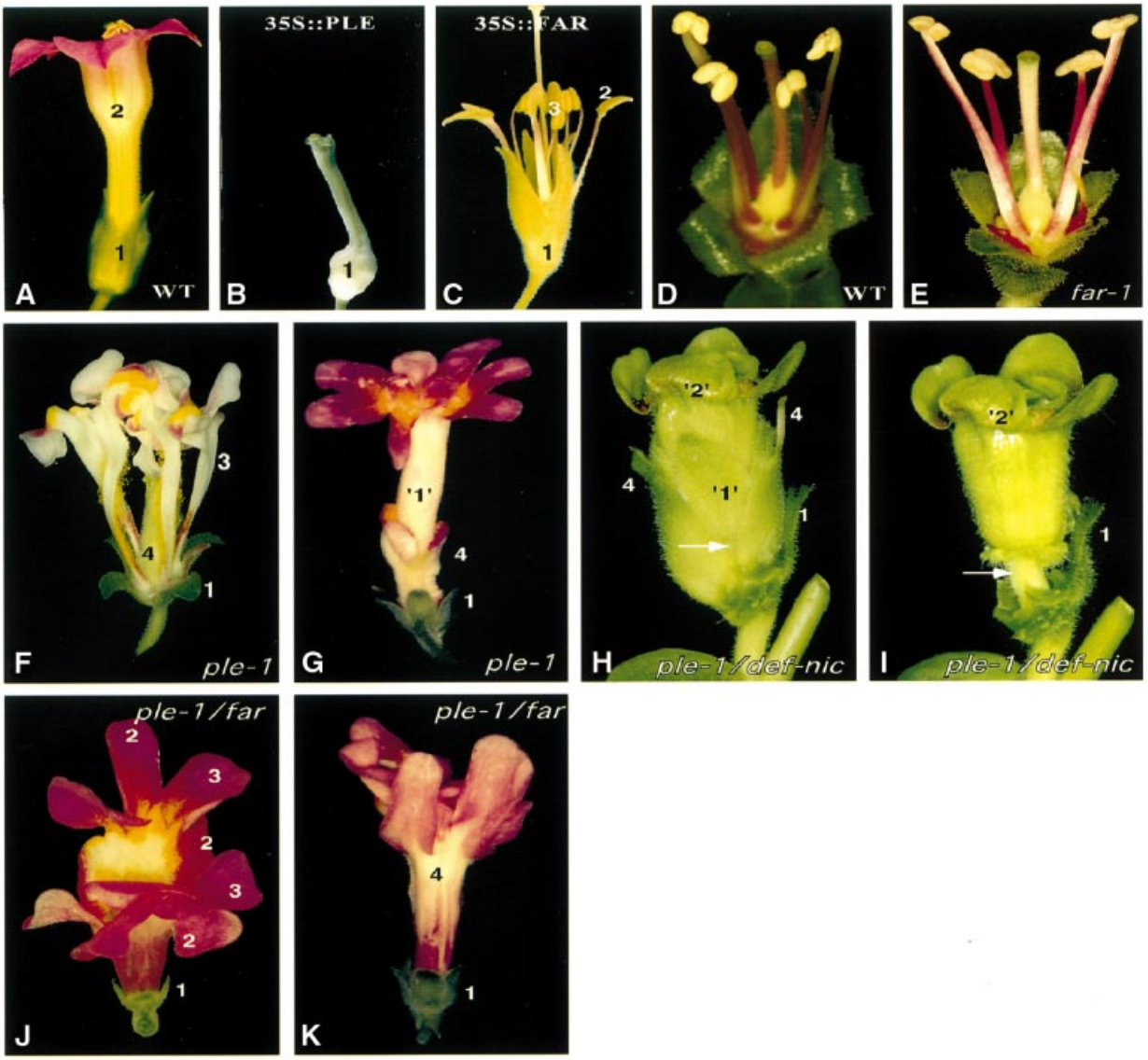

Fig. 3. Phenotypic analysis of ectopic expression of *PLE* and *FAR* in tobacco and loss of *FAR* function in *Antirrhinum* flowers. Representative tobacco flowers are shown from wild-type (**A**), 35S::*PLE* (**B**) and 35S::*FAR* (**C**) plants. The normal anthers of wild-type *Antirrhinum* flowers (**D**) are compared with the empty, slightly shrunken anthers found in *far* mutant flowers (**E**). The phenotype of the *ple*-1 single mutant flowers with the second (**F**) or second and third (**G**) whorls removed to reveal the inner organs. The phenotype of the *ple*-1/*def*-nic double mutant (**H** and **I**) is shown, with the second and third, or second, third and fourth whorls removed to reveal the sepals in the first whorl ('1') and petaloid organs in the second whorl ('2') of the internal flower. The arrow indicates the pedicel inside the fourth whorl. Flowers of the *ple*-1/*far* double mutant are shown without organs internal to whorl 3 (**J**) or with second and third whorls removed (**K**). The whorls are numbered. Flowers in (D), (E) and (F) are shown in front view, (G), (J) and (K) from the back, and (H) and (I) from the side.

to stamens than is seen in 35S::*PLE* flowers (Figure 3C). Pollen collected from second whorl anthers of 35S::*FAR* plants has been used successfully in crosses. The filaments of the second whorl stamens are often flattened and slightly petaloid. No abnormalities were observed in the third and fourth whorls or in the vegetative tissues. These experiments suggested that the two C-function genes might play distinct and complementary roles in flower development, with *PLE* mainly involved in female reproductive organ development and *FAR* mainly involved in the development of male reproductive organs. Representative 35S::*PLE* plants were crossed with 35S::*FAR* plants and the progeny analysed. Purely additive effects were observed in the double transgenics; the plants showed the strong first whorl carpeloidy seen in 35S::*PLE* lines combined with strong second whorl staminoidy typical of 35S::*FAR* plants

Table I. Results of the two-hybrid library screen

Bait	Trans-formants screened	His^+ colonies picked	$LacZ^+$ colonies	Specific interactions
HF7c(BD/FAR)	3.5×10^6	450	33	10

Table II. Testing for interaction between known factors

	PLE	FAR	SQUA	DEFH49	DEFH200	DEFH72	DEF	GLO
PLE	–	–	+	+	+	+	–	–
FAR	–	–	+	+	+	–	–	–

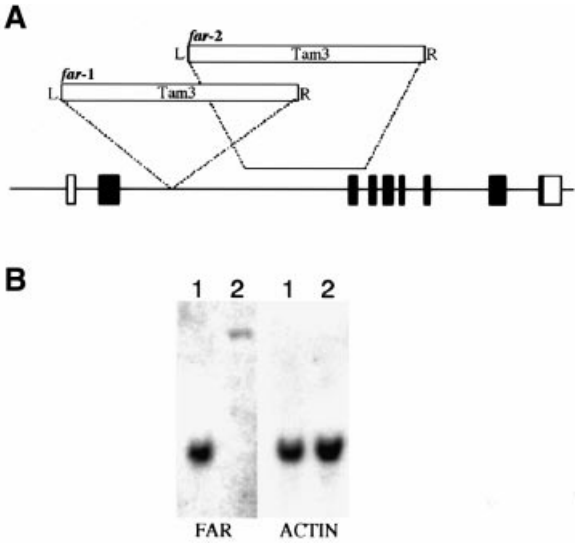

Fig. 4. Transposon insertion mutants in the *FAR* gene. (**A**) A genomic map of the *FAR* gene is shown indicating the sites and orientation of integration of the transposon *Tam3* in each of the two independent mutants *far*-1 and *far*-2. The integration in *far*-2 is associated with a deletion which includes exon 3. (**B**) A Northern blot of mRNA isolated from wild-type (lane 1) and homozygous *far*-2 (lane 2) flower buds was probed with *FAR* and *ACTIN* as a loading control. The large faint upper band in the lane probed with *FAR* probably derives from transcriptional readthrough into the transposon.

(not shown). Whether the phenotypic differences between the 35S::*PLE* and 35S::*FAR* transgenic plants result from the absence of their heterodimerization partners or from their inappropriate expression pattern or altered interaction potential in tobacco with the *Antirrhinum* proteins was not pursued.

Screening for transposon insertions

Sequence analysis and yeast two-hybrid experiments had shown similarity between *PLE* and *FAR*, yet *in situ* and transgenic studies suggested that the two genes may play different roles in flower development. Furthermore, large-scale *Antirrhinum* mutagenesis programmes consistently had failed to produce a C-function-like mutant which was not allelic with *ple*, providing further evidence that the developmental role of *FAR* was not analogous to that of *PLE*. In order to resolve these questions, a reverse genetic PCR-based approach was taken to identify transposon insertions in the *FAR* gene. Pools of DNA (E.Keck, R.Carpenter and E.Coen, unpublished results) were screened by PCR, leading to the identification of two individual plants, *far*-1 (Stock No. JIT2) and *far*-2 (Stock No. JIT3), with insertions of the transposon *Tam3* (Figure 4A). The positions and orientation of the *Tam3* insertions were mapped by PCR using gene-specific primers and primers specific for the right and left borders. In both cases, the orientation of the *Tam3* element was the same, with the left border of the element towards the 5′ end of the *FAR* gene, an orientation which previously has resulted in loss of gene function, even when insertion has occurred within introns (Bradley *et al*., 1993). Both insertions were within the large second intron, with the insertion in *far*-1 being further towards the 5′ end than that in *far*-2. The insertion in *far*-2 is associated with a deletion which has removed part of the coding region (Figure 4A). Southern blot analysis of the two plants confirmed the insertion of foreign DNA into the *FAR* gene (not shown). *far*-2 homozygous plants were crossed with wild-type, the resulting F_1 plants were selfed and co-segregation of the *far* mutant phenotype with the *far*-2 mutant allele was observed in the segregating F_2 population grown at low temperature. Loss of expression of the *FAR* gene was shown for the homozygous *far*-2 plants by Northern blot analysis (Figure 4B). In *far*-2 mutant plants, the *FAR* transcript is undetectable, although the actin control demonstrates the integrity of the RNA.

Phenotype of far mutants

The two *far* mutant plants showed identical developmental abnormalities and, therefore, only *far*-2 was used for further morphological and genetic studies. In contrast to our expectations, homeotic changes were not observed and the plants developed normally. Close inspection of the mutant flowers revealed a variable degree of male sterility. The anthers of homozygous *far* mutant plants, particularly when grown under warmer conditions, appeared white (at early stages) or brown (at later stages) and were slightly shrunken (Figure 3E). At lower temperatures, some pollen was formed in the mutant anthers but always less than was seen in wild-type control plants. Pollen development appears normal up to the early stages of microspore development when callose begins to surround the microspores. After this, a variable range of defects appear, ranging from apparently normal development to complete degradation of microspores and tapetal cells (not shown). In extreme cases, the locules are either completely empty or contain some empty, microspore-like structures surrounded by a filamentous material, which might be the remains of degenerated tapetal cells. Occasionally, normal, fertile anthers were formed on *far* mutant plants. When this happened, it was usually the upper pair which were normal, whilst the lower pair often remained empty, suggesting that the developmental process controlled by *FAR* is also influenced by the floral symmetry genes (Luo *et al*., 1996).

The male-sterile phenotype of *far* plants is similar to a pre-existing *Antirrhinum* mutant, *albidiflora* (*albi*). In order to test whether *albi* is also caused by a defect in *FAR*, the *FAR* gene was PCR amplified from homozygous and heterozygous *albi* plants and wild-type sisters, all of which were derived from selfing the progeny of a cross between *albi* (in a Si50 background) and the wild-type 165E. A *FAR* polymorphism was detected by digesting the resulting PCR fragment with the enzyme *Alu*I. The polymorphism did not co-segregate with the *albi* mutation, showing that, although similar in phenotype, *albi* and *far* are unlinked (data not shown). Thus, *far* represents a novel male-sterile mutant.

Expression of PLE in far mutant plants

Since *far* mutants appear morphologically normal, with the exception of empty anthers, it was likely that *PLE* expression would also be normal in *far* mutants. However, *in situ* hybridization experiments revealed that in the absence of *FAR*, the expression pattern of *PLE* expands. *PLE* expression, which is confined to the region of the stomium in the mature wild-type anthers (Figures 5A and

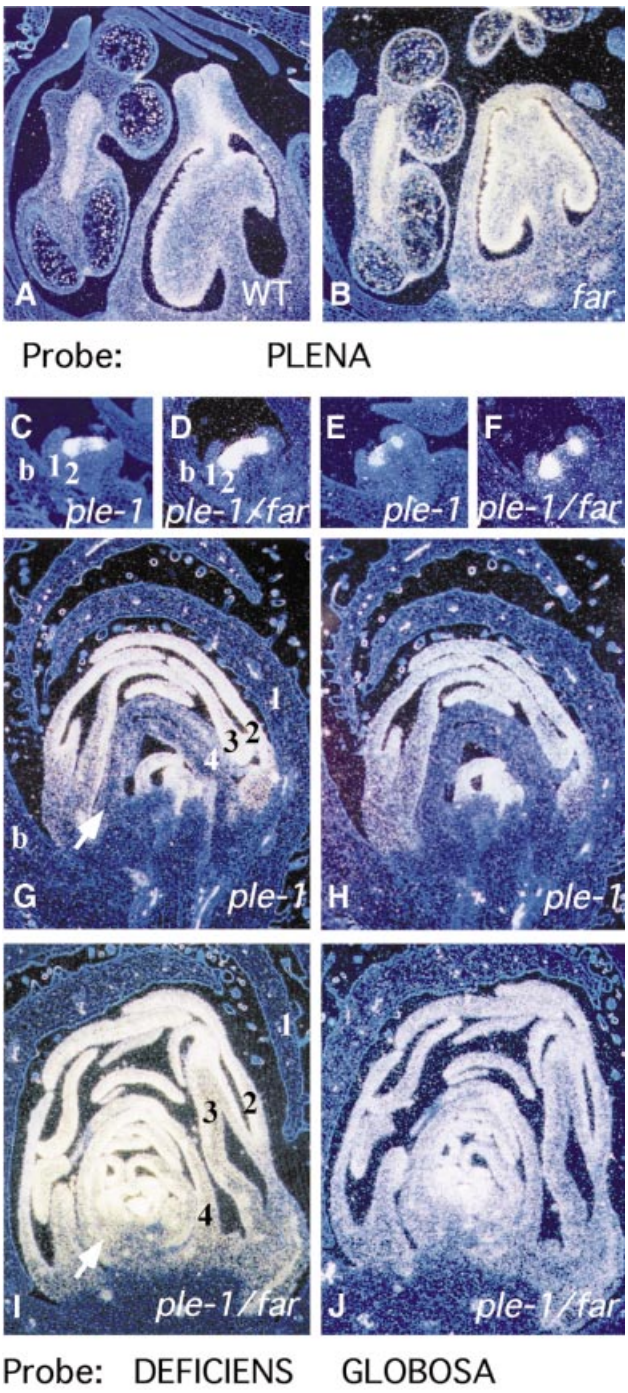

Fig. 5. Comparison of the *in situ* expression patterns of *PLE*, *DEF* and *GLO* in wild-type and mutant flowers. Sections of mature wild-type and *far* mutant buds were probed with *PLE* (**A** and **B**). Serial sections of early and late stages of *ple* and *ple*/*far* mutant flower buds were probed with *DEF* (left hand column, C, D, G and I) and *GLO* (right hand column, E, F, H and J). The stages used were: stage 4 (**C**–**F**) and late stage 6 (**G**–**J**). Whorls are numbered as in Figure 2.

2E), can now be detected throughout the anther, in a pattern which looks like the superimposition of the expression patterns of *FAR* and *PLE* (compare Figures 2E and F, and 5B). Similarly, in the carpel, expression of *PLE*, which had been detectable mainly around the developing ovules in the wild-type (Figures 5A and 2E), is now much stronger and spread throughout the carpel wall and placenta (Figure 5B). This increase and expansion of the *PLE* expression pattern in *far* mutants suggests that *FAR* negatively regulates *PLE*. It is possible that in *far* mutants the expansion of the *PLE* expression domain, into tissues which would normally express *FAR* in wild-type flowers, compensates for the loss of *FAR* activity.

Genetic analysis of far mutants

ple-1/*far* double mutants were constructed and analysed. Superficial examination of the double mutants suggested that *ple*-1 is epistatic to *far* since, like *ple* (Bradley *et al*., 1993), *ple*-1/*far* flowers are indeterminate and show homeotic conversion of reproductive organs to perianth organs. However, more detailed investigation of the organs produced in the third and fourth whorls of *ple*-1 and *ple*-1/*far* flowers revealed a surprising interaction between the two genes. The third whorl of *ple*-1 mutant flowers consists of laterally non-fused petaloid organs which resemble stamens in that their lower part is filament-like and narrow and the upper part is slightly broadened and flag-like (Figure 3F). In contrast, the upper part of the third whorl organs of the *ple*-1/*far* double mutant are broader and the organs fully or partially fuse to the second whorl at their lower part, similarly to a *ple* null mutant (Figure 3J). This more pronounced petaloid morphology of *ple*-1/*far* double mutants compared with *ple*-1 mutants suggests that although *FAR* is unable to compensate for the loss of *PLE*, it still plays some positive role in stamen organ identity.

A more dramatic effect of *FAR* on organ identity is observed in the fourth whorl. In *ple*-1 mutants, the degree and kind of homeotic fourth whorl transformation is variable (Figure 3F and G), but usually two to three sepaloid/petaloid/carpeloid organs are formed. Within this whorl, another mutant flower develops to a variable extent (Figure 3G), initiated by slightly sepaloid petals composed of a mixture of petal and sepal tissue. This sepaloid feature is observed more easily in the double mutant between *ple*-1 and a weak allele of *deficiens*, *def*-nic (Schwarz-Sommer *et al*., 1992). In the *ple*-1/*def*-nic double mutant, the fourth whorl consists of two sepaloid carpels and, internal to them, borne on a pedicel, a new flower is initiated by a whorl or spiral of five or more sepals (Figure 3H and I), followed by petals with morphological features characteristic of *def*-nic second whorl organs (Figure 3I). In contrast, the fourth whorl of *ple*-1/*far* mutant flowers consists of four to five petals forming a tube and corolla similar to the second whorl petals, followed by further spirally arranged petals (Figure 3K). Thus, in the double mutant, both the identity and the number of organs produced in the fourth whorl are altered.

Since petal organ identity in *Antirrhinum* is determined by the expression of the B-function genes *DEF* and *GLO*, it seemed likely that the conversion of the fourth whorl to petals in *ple*/*far* double mutants resulted from ectopic expression of *DEF* and *GLO*. To test this, *in situ* experiments were carried out on *ple* and *ple*/*far* double mutant flowers using *DEF* and *GLO* as probes. At stage 4, expression of both *DEF* and *GLO* in *ple*-1 buds is similar to that normally observed in wild-type buds (Figure 5C and E). At the same stage in the *ple*-1/*far* double mutant, expression of both genes is markedly stronger, although the tissue specificity is maintained; *GLO* is confined mainly to the developing second and third whorls (Figure 5F) and *DEF* is clearly detectable in the inner three whorls

(Figure 5D). In mature *ple*-1 flower buds, the expression pattern is maintained, *DEF* being expressed mainly in the second and third whorls, reduced in the fourth whorl (arrowed in Figure 5G) and increased again in internal whorls. Expression of *GLO* in mature *ple* buds is similar to that of *DEF*, but as in younger stages, *GLO* transcripts are less detectable in the fourth whorl than are those of *DEF* (Figure 5H). In the *ple*-1/*far* double mutant, the reduced expression of *DEF* and *GLO* in the fourth whorl is no longer apparent and both transcripts are strongly expressed in the organs internal to the first whorl (Figure 5I and J, fourth whorl arrowed in I). Thus, *PLE* and *FAR* act independently to prevent the expression of the B-function in the fourth whorl.

Discussion

The developmental role of FAR

In *Antirrhinum*, two MADS-box genes, *PLE* and *FAR*, share typical features of C-function genes, including gene organization, sequence similarity and protein–protein interaction potential. In *ple* mutant flowers (Bradley *et al*., 1993), the reproductive organs are converted homeotically to perianth organs, and further perianth organs develop inside the fourth whorl (Figure 3F and G). *PLE* is therefore required to specify the identity of the stamens and carpels and to confer floral determinacy. In the newly isolated *far* mutant, the only apparent phenotype is a failure to complete normal pollen development. Analysis of *ple*/*far* double mutants reveals an unexpected interaction between the two genes and demonstrates that they are partially redundant. In *ple* single mutants, the fourth whorl develops as two sepaloid/carpeloid/petaloid organs (Figure 3G). In contrast, the fourth whorl organs of *ple*/*far* double mutants develop as four or five well-formed petals (Figure 3J and K). Furthermore, the B-function genes, *DEF* and *GLO*, which normally are mostly excluded from the fourth whorl, become ectopically expressed there in the *ple*/*far* double mutant (Figure 5I and J). Since the B-function genes are largely confined to the second and third whorls in both *ple* and *far* single mutants, it seems that *PLE* and *FAR* are redundant with respect to their ability to exclude the B-function genes from the fourth whorl. In addition, combination of a weak *ple* allele with *far* enhanced third whorl petaloidy, suggesting a role for *FAR* in the control of wild-type stamen identity.

The relative contribution of each gene to the development of the reproductive organs is more difficult to assess. *FAR* is incapable of substituting for the organ identity function of *PLE*, since no reproductive organs are formed in *ple* mutants even though *FAR* is expressed. *FAR* is involved in pollen development, but the small amount of pollen which occasionally is produced in *far* mutant anthers shows that it is not absolutely required. In contrast, *PLE* can direct the formation of male and female reproductive organs in *far* mutants, although the anthers are frequently empty. It could be that *PLE*, perhaps indirectly, leads to the production of the limited amount of pollen found in *far* mutants. Conversely, it could be the expansion of *PLE* expression within the developing anthers of *far* mutants which is responsible for the failure to undergo normal pollen development. The phenotypic effect of the loss of *FAR* function could be limited because *PLE* expression expands in *far* mutants. In contrast, in *ple* mutants, *FAR* expression decreases and cannot compensate for the loss of *PLE*. This dependence of *FAR* on the expression of *PLE* is circumvented in transgenic plants where *FAR* is controlled by the 35S promoter. Indeed, ectopic expression of *Antirrhinum FAR* in transgenic tobacco plants is sufficient to cause the development of stamens in the second whorl of tobacco flowers. Preliminary results with transgenic *Antirrhinum* plants confirm this, although the extent of homeotic transformation of the first and second whorls is less pronounced (I.Heidmann, Z.Schwarz-Sommer and B.Davies, unpublished). In summary, *FAR* appears capable of functions similar to those of *PLE*, but this is not apparent in the single mutants because of the regulatory interactions between the two genes.

From sequence to function

Various criteria have been used to assign MADS-box genes to sub-groupings within the family. These include overall sequence similarity, the presence of specific amino acid residues at characteristic positions within the MADS-domain, the length of the I-region, the presence or absence of an N-terminal extension and a high degree of amino acid sequence identity amongst the five or six C-terminal amino acids. It has been supposed that some of these indicators could be used to suggest functions for newly discovered MADS-box genes (Theissen *et al*., 1996). In the case of *FAR*, these indicators, together with the expression pattern, suggested a typical C-function gene. Ectopic expression experiments supported this, since homeotic conversions of petals to stamens were observed, although the more typical sepal to carpel conversions were not apparent. Yeast two-hybrid screens for proteins capable of interacting with FAR resulted in the isolation of almost exactly the same subset of cDNAs as was identified when the known C-function factor PLE was used in similar experiments. From our understanding of the C-function, it would therefore be expected that a loss-of-function *far* mutant should result in homeotic conversion of the perianth to reproductive organs and a loss of floral determinacy. In fact, the *far* single mutants showed no homeotic changes and no effects on determinacy. The defect in male fertility, which is the only change apparent in *far* mutants, could not have been predicted from either the sequence classification of *FAR* or from any of the subsequent biochemical experiments. It also could not have been predicted that mutation of the *FAR* gene would lead, in a *ple* mutant background, to alterations in organ identity and number in whorls which are unaffected in *far* single mutants. The complications which obscured the developmental role of *FAR* in *Antirrhinum*, redundancy and regulatory interaction, have been observed for other MADS-box genes (Bowman *et al*., 1993; Liljegren *et al*., 1998) and demonstrate that the developmental role of a gene is dependent on both its primary sequence and its position within the regulatory hierarchy. The implication of this result is that it will be impossible to determine the developmental role of other members of the MADS-box gene family unless the appropriate mutants and mutant combinations are first isolated. Caution will be needed when interpreting the effects of ectopic expression of MADS-box genes, especially when inferring their functions from heterologous

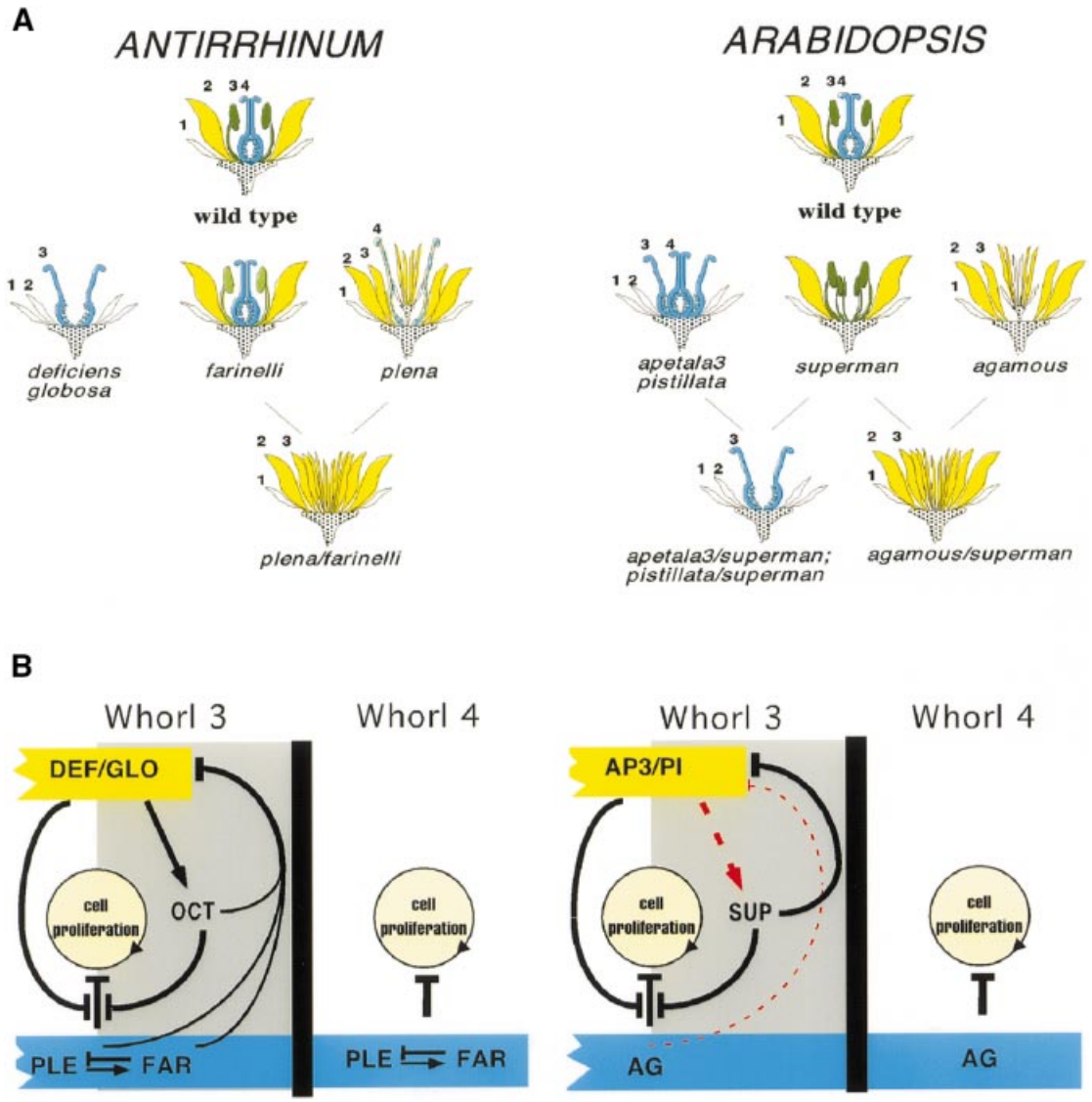

Fig. 6. Differences between homeotic mutants of *Antirrhinum* and *Arabidopsis*. (**A**) A comparative schematic representation of the mutants and double mutants of *Arabidopsis* (right) and *Antirrhinum* (left) referred to in the text. The whorls are numbered to highlight the differences between the *Arabidopsis* and *Antirrhinum* B-function mutants (*pistillata*, *apetala3*, *globosa* and *deficiens*) and C-function mutants (*agamous* and *plena*). The mutant phenotypes are described as follows: *Arabidopsis ag* (Bowman *et al*., 1991), *pi* (Jack *et al*., 1992), *ap3* (Goto *et al*., 1994), *sup*, *sup*/*ag*, *sup*/*ap3*, *sup*/*pi* (Schultz *et al*., 1991; Bowman *et al*., 1992), and *Antirrhinum ple* (Bradley *et al*., 1993; this report), *def*, *glo* (Schwarz-Sommer *et al*., 1992; Tröbner *et al*., 1992), *far*, *ple*/*far* (this report). (**B**) A model to explain the observed differences in phenotypes in terms of regulatory gene activity in the third and fourth whorls. B-function expression is shown in yellow, *SUP* (*OCT* in *Antirrhinum*) is shown at the boundary of the third whorl in grey and C-function expression (*AG* in *Arabidopsis*, *PLE* and *FAR* in *Antirrhinum*) is shown in light blue. The barred lines leading from the C-functions towards the circle indicate that the C-function represses production of a new whorl and causes termination. The barred lines leading from the B-function and from *SUP*/*OCT* indicate that they act to prevent C-function-dependent termination. The arrow and barred line between *PLE* and *FAR* indicate that *PLE* activates *FAR* and *FAR* represses *PLE*. Arrows and barred lines are not meant to imply direct interaction. Two key differences in the regulatory control of *OCT* in *Antirrhinum* and *SUP* in *Arabidopsis* are proposed. First, unlike *SUP*, *OCT* expression is wholly dependent on the expression of the B-function. This is shown by an arrow leading from B to *OCT* in *Antirrhinum* and a dotted red line leading from B to *SUP* in *Arabidopsis*. Secondly, in *Antirrhinum*, the negative regulation exerted by *OCT* on the B-function can only occur in the presence of the C-function *PLE* or *FAR*. This is indicated by barred lines running from both *PLE* and *FAR* and joining lines from *SUP* to the boundary of the B-function expression domain. These two regulatory changes allow the model to predict the phenotypes of all the single and double mutants shown in (A) and discussed in the text. The thin red barred line leading from *AG* to the B-function represents a potential minor regulatory interaction.

systems. As a consequence, reverse genetic strategies such as the one used in this report will be required to understand the contribution of these genes to plant development.

Control of floral determinacy in different species: beyond the ABC model?

Although the *Antirrhinum* and *Arabidopsis* C-function mutants *ple* and *ag* both consist of indeterminate whorls of perianth organs, they differ with respect to the identity and organization of certain whorls. In *ple*-1 flowers, the fourth whorl consists of two to three organs which surround a new flower, borne on a pedicel and initiated by sepaloid organs (Figure 6A). A new flower also develops in the *Arabidopsis ag* mutant, but in this case the four sepaloid organs of the first whorl of the internal flower form inside the third whorl (Figure 6A). Thus, in *Arabidopsis*, the new internal flower of the C-function mutant is initiated after three whorls, not after four whorls as in *Antirrhinum*.

In this interpretation of C mutant phenotypes in either species, there is no homeotic transformation of carpels to sepals, as proposed in the ABC model (see Introduction), but rather initiation of a new flower, albeit at a different site, as shown above and discussed below. The different phenotypes of the two C-function mutants become identical when they are combined with other mutants. In *ple*/*far* double mutants, there is no initiation of a new internal flower (Figure 6A); all internal whorls consist of petals. This is similar to the phenotype of the double mutant between *ag* and *superman*/*flo10*, which also fails to initiate a new internal flower and consists of sepals surrounding multiple whorls of petals (Schultz *et al*., 1991; Bowman *et al*., 1992). *SUPERMAN*/*FLO10* (*SUP*) acts at the boundary of whorls three and four to prevent expression of the B-function in the fourth whorl, probably by controlling the proliferation of B-containing cells (Sakai *et al*., 1995). We show here that in *Antirrhinum* the B-function is negatively regulated by *PLE* and *FAR*.

An analogous phenotypic difference is apparent when the B-function mutants of *Antirrhinum* (*def* and *glo*) and *Arabidopsis* (*ap3* and *pi*) are compared. In the *Antirrhinum* B-function mutants, no fourth whorl organs develop due to the antagonistic effect on determinacy between the B- and C-functions (Tröbner *et al*., 1992); the B-function prevents C-function-dependent termination of development (Figure 6A). In the *Arabidopsis* B-function mutants, a fourth whorl develops, indicating that, unlike in *Antirrhinum*, the mechanism to prevent premature termination by the C-function must be at least partially independent of the B-function (Figure 6A). It has been reported that double mutants between *sup*/*flo10* and *pi* or *ap3* produce only three whorls (Schultz *et al*., 1991), making them more similar to the *Antirrhinum* single B-function mutants *def* and *glo* (Figure 6A). The fact that *sup*/*flo10* single mutants produce a proliferation of stamens within the third whorl shows that *SUP* and the B-function act together to prevent C-function-dependent termination. Taken together, these observations suggest that initiation of fourth whorl primordia and subsequent termination of flower development in the centre of the flower are controlled differently in the two species and indicate that the difference could centre on the role of *SUP*.

A possible explanation for the observed differences in mutant phenotypes described above could be that *Antirrhinum* lacks a *SUP* function and instead uses *PLE* and *FAR* to regulate the B-function negatively. However, the role of *SUP* in *Arabidopsis* cannot be taken wholly by *PLE* and *FAR* in *Antirrhinum* because the B-function is expressed in the third whorl, although both *PLE* and *FAR* are active there, suggesting that *PLE* and *FAR* co-operate with a spatially restricted function at the boundary of whorls three and four and do not act independently to prevent B-function expression there. A mutant with a similar phenotype to *sup*/*flo10* has been found recently in *Antirrhinum* and named *octandra* (*oct*) (R.Carpenter, personal communication), suggesting that *OCT* performs the function of a *SUP*-like gene in *Antirrhinum*. In order to explain the observed differences between *Antirrhinum* and *Arabidopsis* mutant phenotypes, we need to postulate that *OCT* differs from *SUP* in two key ways. First, *OCT* requires *PLE* or *FAR* in order to exclude the B-function from the fourth whorl, whereas *SUP* can act independently of *AG*. This is supported by the fact that the B-function does not expand into the fourth whorl of *ag* mutants (Jack *et al*., 1992) but does in *ple*/*far* double mutants (this report). Secondly, *OCT* expression or function is more dependent on the B-function in *Antirrhinum* than is *SUP* in *Arabidopsis*, although other factors must also be involved to control the expression of *OCT* at the boundary between the third and the fourth whorl. Figure 6B illustrates how these differences in the regulation of and by *OCT*/*SUP* could account for all the observed wild-type and mutant phenotypes of *Antirrhinum* and *Arabidopsis*. For simplicity and clarity, Figure 6B and the following paragraphs do not consider that factors other than SUP/OCT and the organ identity controlling functions are involved in the control of organ initiation and determinacy and that the regulatory relationships are likely to be indirect and more complex.

In the fourth whorl of *Antirrhinum* and *Arabidopsis*, only the C-function, controlled by *PLE*/*FAR* or *AG*, is expressed (Figure 6B). This leads to the formation of carpels and termination of floral development. In the third whorl of *Arabidopsis*, the co-expression of the B-function genes, *AP3* and *PI*, with *AG* leads to the production of stamens, whilst *AP3*/*PI* and *SUP* prevent *AG* from terminating flower development in this whorl. Similarly, in *Antirrhinum*, *DEF*/*GLO* and *OCT* prevent *PLE* or *PLE*/*FAR* from terminating floral development in the third whorl. If *OCT* expression in *Antirrhinum* is dependent on *DEF*/*GLO*, *OCT* will not be expressed in *def* and *glo* mutants and development will terminate in the third whorl because there is no *DEF*/*GLO* or *OCT* activity to counteract the terminatory influence of *PLE* or *PLE*/*FAR*. In contrast, *Arabidopsis ap3* and *pi* mutants produce four whorls because *SUP*, being partly independent of *AP3*/*PI*, will still act to prevent *AG*-dependent termination in the third whorl. In *ap3*/*sup* and *pi*/*sup* double mutants, termination occurs with the production of third whorl carpels because, as in *Antirrhinum def* and *glo* mutants, both the B-function and *SUP*/*OCT* are absent.

In *Arabidopsis ag* mutants, *AP3* and *PI* remain excluded from the fourth whorl due to the activity of *SUP*. Thus, in the fourth whorl, there is no C- or B-function activity and a new flower is produced. In *Antirrhinum ple* mutants, *DEF* and *GLO*, which we suggest to be under the control of *PLE*/*OCT* and *FAR*/*OCT*, expand slightly into the fourth whorl as a result of a loss of *PLE* and a concomitant reduction in *FAR*. Some residual control over *DEF* and *GLO* is maintained in *ple* mutants due to the reduced activity of *FAR*/*OCT*. The slight expansion of *DEF* and *GLO* into the fourth whorl is sufficient to prevent the initiation of a new mutant flower until after initiation of the fourth whorl. Loss of *FAR* does not have similar consequences because in the absence of *FAR*, *PLE* expression increases to compensate. In contrast, the double mutants *ag*/*sup* and *ple*/*far* show the same phenotype because in neither case is the B-function prevented from being expressed in the fourth whorl. The model thus predicts that OCT expression or function is absent in the *def* and *glo* mutants and that these *Antirrhinum* B-function mutants would produce four whorls if an *OCT* transgene were to be expressed in the third whorl. This hypothesis can be tested when *OCT* is isolated from *Antirrhinum*. It also predicts that the concommitant loss of *ple*, *far* and

oct will not confer changes to floral morphology other than those observed in the *ple*/*far* double mutant.

Materials and methods

cDNA libraries, in situ hybridization and Northern blotting

In situ and Northern blot expression analysis and cDNA library construction and screening were carried out as previously described (Sommer *et al*., 1990; Huijser *et al*., 1992). Photographs were taken in dark field to detect the silver grains and superimposed on calcofluor-stained epifluorescence to visualize the underlying tissue. Sequencing was carried out using the fmol cycle sequencing system (Promega) with sequence analysis by MacVector (Oxford Molecular Group).

Two-hybrid screening

The predominant *FAR* cDNA was amplified using oligonucleotide primers to introduce cloning sites. Amplification was carried out using the oligonucleotide primers 5′-ATAGGAATTCGCGTCTCTAAGCGATC-3′ and 5′-GGAAGGATCCATTTTCTCCAAGCGCC-3′ followed by digestion with *Eco*RI and *Bam*HI and insertion into pGBT9 to form the bait. The bait included all the *FAR* coding sequences excluding the initiation codon. Two-hybrid screens were carried out as previously described (Davies *et al*., 1996b). Interactions were investigated both by screening an *Antirrhinum* floral cDNA yeast expression library and by directly testing for interaction between the FAR bait and previously isolated MADS-box preys.

PCR pools screening

The reverse genetic screen used here was established at the John Innes Centre, Norwich, UK (E.Keck, R.Carpenter and E.Coen, unpublished). Integrations of the transposable element *Tam3* into the *FAR* gene were detected by hybridization of the *FAR* probe to blots of PCRs containing a gene-specific primer, a *Tam3*-specific primer and several pools of genomic DNA from defined sets of plants. Two DNA pools from the John Innes Institute, Norwich, UK, were screened for integrations into individual plants. The two pools were from the years 1992 and 1993 and were derived from 6350 and 6300 plants, respectively. The pools were screened as 31 superpools of 450 plants, each of which was created by combining three subpools of genomic DNA prepared from the leaves of 150 plants. Positives were first confirmed on the subpools of 150 plants and subsequently on 10 new subpools generated from batches of 15 separately collected leaves from those plants which contributed to the positive subpool. Finally, seed collected from the 15 plants in each positive pool was sown and the seedlings were tested to confirm integrations into *FAR*.

From the genomic sequence of *FAR*, four oligonucleotide primers were chosen to match approximately the length and base composition of the two primers specific for the left and right borders of the *Antirrhinum* transposable element *Tam3*. The primers used were: FAR1F, 5′-CGTTTCTATTTTTGACTCTATGG-3′; FAR4F, 5′-CCATCCTCTTTGACAGTTCC-3′; FAR4R, 5′-GAGGATGGACAAATGAGAAC-3′; FAR7R, 5′-GATTTAGTTTGTGTGCGATTC-3′; Tam3R, 5′-CTCGGCACGTTTCACATCTTTA-3′; and Tam3L, 5′-CACGGCCCAATTCACATCTTTA-3′.

PCR was carried out in 50 μl reactions, and 5–10 μl of each superpool reaction was dot blotted onto a nylon membrane using an eight channel micropipette and the filter was baked and hybridized with a *FAR* probe. Subsequent subpool screening was by Southern blotting of the PCR products.

Genetic stocks and transgenic plants

Antirrhinum majus plants were grown in a greenhouse at 18–25°C with additional light during winter. A climate chamber at 15°C was used for growth at lower temperatures. The following mutant and wild-type lines were used in this study: *ple*-1 and *def*-nic (Stubbe, 1966) from the Gatersleben seed collection, *Ple*-888 (Macho) (Schwarz-Sommer *et al*., 1990; Lönnig and Saedler, 1994), *albidiflora* Gatersleben, Sippe50 (Stubbe, 1966) and Gatersleben, 165E (*niv-98::Tam3*) Rosemary Carpenter, Norwich. *ple*/*far* double mutants were isolated by selecting plants in the F_2 of a cross using *ple*-1/*ple*-1 flowers, which occasionally produce some pollen, to pollinate *far*-2/*far*-2 plants. The double mutants were identified by selecting for a *ple* phenotype and testing for homozygosity of *far* by PCR. *Ple*-1/*def*-nic double mutants were obtained in the selfed progeny of plants heterozygous for both mutant alleles, produced by crossing a *def*-nic plant with pollen from *ple*-1. Mutants and double mutants were identified by their phenotype and segregation ratio (*ple*-1:*def*-nic:*ple*-1/*def*-nic = 28:22:5). In addition, plants with elongated internodes and pedicels segregated in this population together with the decrease of the apical dominance, most likely due to the presence of a trait carried by the *def*-nic line. Segregation of this trait conferred considerable variation to the mutant phenotypes, revealed by the presence or absence of an internal pedicel when *ple*-1 was mutant, and by decreased or enhanced male features of *def*-nic flowers.

Tobacco transformation was as previously described (Davies *et al*., 1996a). The *FAR* cDNA was amplified by PCR, cloned as a transcriptional fusion behind the cauliflower mosaic virus (CaMV) 35S promoter into the binary vector pPCV701, and the insert sequenced. Following agrobacterial-mediated transformation of tobacco, 26 kanamycin-resistant plants were isolated, 18 of which showed clear morphological changes. Selection for 35S::*PLE* and 35S::*FAR* transformants and for the double transgenic was on hygromycin, kanamycin or both antibiotics, respectively. All abnormalities segregated as dominant traits.

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